THYMUS-INDEPENDENT (B) CELL PROLIFERATION IN SPLEEN CELL CULTURES OF MOUSE RADIATION CHIMERAS STIMULATED BY PHYTOHEMAGGLUTININ OR ALLOGENEIC CELLS*

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In the course of experiments studying the mitotic response to various agents of spleen cell cultures obtained from mouse radiation chimeras bearing chromosomally marked thymus-derived (T) cells (1), it was observed that stimulants such as phytohemagglutinin (PHA) and allogeneic cells, usually considered to generate only a T cell response (2, 3), also induced a striking, though more protracted, B cell proliferation.

Materials and Methods

To prepare chimeras with chromosome markers (1), CBA/Ca or CBA/H-T6T6 mice were thymectomized at 4–8 wk of age, subjected 2–4 wk later to 850–950 rads of total body irradiation, and injected within 24 hr with 5×10^6 bone marrow cells treated with mouse AKR anti-θC3H serum with rabbit complement (C). Within 4–8 days, $4–8 \times 10^7$ thymocytes (from 4–6-wk old donors) were injected intravenously. Three types of CBA/Ca $\rightarrow$ CBA/H-T6T6 chimeras were prepared by reconstitution after irradiation: (*a*) with bone marrow of the host karyotype and thymocytes of the other karyotype (T/H + BM mice, i.e. mice where injected thymocytes have one karyotype, and bone marrow cells and residual host cells have the other); (*b*) with bone marrow of the other karyotype and thymocytes of the host karyotype (T + H/BM mice); (*c*) with bone marrow of (CBA/Ca $\times$ CBA/H-T6T6)F_1 hybrids (bearing a single T_6 chromosome) and thymocytes of the other karyotype (T/H/BM mice, i.e. mice where three karyotypes can be found, thymocytes and residual host cells bearing respectively two T_6 chromosomes or none, and bone marrow cells only one T_6). 4–8 wk after reconstitution, cell suspensions were prepared from the spleen and 2×10^6 viable nucleated spleen cells distributed to each culture tube in 1 ml of medium RPMI 1640 containing 6% fetal calf serum, or 6% fresh decomplemented Wistar rat serum. Parallel cultures were used for evaluation of DNA synthesis and karyotypic analyses (4) (usually 50 mitoses analyzed per culture, with a minimum of 30 under less favorable conditions).

RESULTS AND DISCUSSION

PHA-Stimulated Cultures.—PHA-P (Difco Laboratories, Inc., Detroit, Mich.; 0.5%) or a saline extract of Phaseolus vulgaris (an experimentally

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determined optimal amount) was added to the cultures. A peak of DNA synthesis was observed between days 2 and 3, with marked stimulation still present on days 4 and 5. Study of the karyotypes showed a strong predominance of T mitoses on days 2 and 3, while most mitoses were of BM type on days 4 and 5

TABLE I

Karyotypic Analysis of Spleen Cell Cultures Stimulated by PHA or Allogeneic Cells

Stimulant and type of experiment	Culture arrested on							
	Day 2		Day 3		Day 4		Day 5	
	% T mit.*	S.I.‡	% T mit.*	S.I.‡	% T mit.*	S.I.‡	% T mit.*	S.I.‡
(*1*) PHA: average results§	79 [6] (72–88)	8–10	77 [17] (58–96)	10–12	26 [7] (4–49)	6–7	8 [4] (2–10)	3
(*2*) PHA: culture of a single chimera T + H/BM	88	8	78	9	24	6.5	6	3
(*3*) MLC of nonimmunized chimeras: average results‖	N.D.		75 [13] (60–96)	3–6	38 [9] (20–53)	2–4	16.5 [4] (6–37)	2
(*4*) MLC of immunized chimeras: average results¶	60 [6] (36–87)	2–3.5	64 [11] (37–90)	3–7	24 [9] (4–58)	2–4.5	10 [5] (0–28)	2
(*5*) MLC of a single chimera T/H + BM imm. with C57 cells s.c. 6 days earlier								
(*a*) with F_1 (T6 × CBA/J) spleen cells	N.D.		74**	3	29	2	N.D.	
(*b*) with F_1 (T6 × C57) spleen cells	N.D.		43**	6	4	4	N.D.	
(*6*) MLC of a single chimera T + H/BM imm. with CBA/J cells s.c. 6 days earlier								
(*a*) with F_1 (T6 × C57) spleen cells	N.D.		75	3	20	N.D.	17	2
(*b*) with F_1 (T6 × CBA/J) spleen cells	N.D.		78	5	20	N.D.	N.D.	

* Numbers in parentheses indicate values observed; numbers in brackets indicate numbers of experiments.

‡ S.I. = stimulation index, or ratio of radioactivity incorporated in stimulated/"background" cultures. For MLC, incorporation of background cultures was calculated as one-half of the sum of incorporations of 2×10^6 chimera spleen cell culture and 2×10^6 F_1 spleen cell culture.

§ Of 34 cultures stimulated by PHA, 14 were from T + H/BM chimeras and 20 from chimeras with three markers. In this last group, the largest number of host mitoses was on day 3 and averaged 11% (0–22); they were counted as T mitosis. Cultures of eight of these chimeras were arrested on sequential days.

‖ 13 chimeras (7 T/H + BM and 6 T + H/BM) were used for MLC with F_1 (T6 × C57) (8) or (T6 × CBA/J) (5). C57BL/6 are *H-2*^{b}, CBA/J *H-2*^{k}, and CBA/Ca not *H-2*^{k} (J. Staats. 1968. *Cancer Res.* **28**:391). MLC of nine of these chimeras were arrested on sequential days. Seven of these chimeras had been immunized with spleen cells of C57 or CBA/J (see results under "immunized chimeras") to compare "primary" and "secondary" types of MLC.

¶ 11 chimeras (4 T/H + BM and 7 T + H/BM) had been immunized with C57 or CBA/J spleen cells by either subcutaneous injection (7) 6 or 8 days earlier or intraperitoneal injection 4 days earlier. MLC of nine of these chimeras were arrested on sequential days.

** Culture of this chimera with PHA gave 82% T mitoses on day 3.

(Table I, experiments 1). This phenomenon was observed in all cultures of the three types of chimeras, as well as in cultures of individual chimeras studied on consecutive days (Table I, experiments 2). Analysis of T/H/BM chimeras showed, especially on day 3, some host mitoses (see legend Table I). Since Doenhoff et al. (2) have described the survival of some host T lymphocytes after lethal radiation, these mitoses were classified as T mitoses. T + H/BM chimeras gave results closely comparable with those of chimeras with three markers where host mitoses were counted as T. If most host lymphoid cells surviving radiation are indeed T cells, T + H/BM chimeras are more accurate to study the T or BM nature of the dividing cells. In 16 cultures with T/H + BM chimeras (not shown on Table I), the average percentage of T mitoses on day 3 was about 10% lower than with the other types of chimeras. The non-stimulated cultures showed an average of 27% T mitoses on day 3 (16 experiments) and 35% on day 4 (8 experiments).

Mixed Lymphocyte Culture (MLC) with Chimeras Not Previously Immunized with Allogeneic Spleen Cells.—1×10^6 spleen cells of T + H/BM or T/H + BM chimeras were mixed with 1×10^6 spleen cells of an F_1 hybrid bearing a single T_6 chromosome (CBA/H-T6T6 × C57BL/6 or × CBA/J), thus allowing the distinction between the two types of chimera mitoses and F_1 mitoses. There was almost no stimulation of DNA synthesis on day 2, a peak between days 3 and 4, and definite stimulation still detectable on day 5. A shift from a majority of T mitoses on day 3 to a majority of BM mitoses on days 4 and 5 was observed in all chimeras, including individual chimeras studied on sequential days (Table I, experiments 3).

MLC with Immunized Chimeras.—Chimeras were injected with allogeneic spleen cells subcutaneously 6–8 days or intraperitoneally 4 days before sacrifice. Such conditions are known, in the rat, to give an earlier peak of DNA synthesis in MLC performed with blood (5) or splenic lymphocytes (6). Although DNA synthesis was not determined daily in these cultures so that a precise comparison with "primary" cultures was not possible, it appeared that significant stimulation (2–3.5 times) was already present on day 2, and that the peak of stimulation was usually higher and occurred more often on day 3 than later, decreasing on days 4 and 5. Karyotypic analysis showed heterogeneous results, with some cultures presenting a picture identical with primary cultures, while others had a percentage of T mitoses lower than 40% on days 2 and 3. In seven immunized chimeras, MLC were set up to explore, in parallel to the "secondary' response, a primary response to F_1 cells unrelated to the immunizing cells (Table I, experiments 5 and 6); in three of them there was an earlier and stronger predominance of BM mitoses in the secondary than in the primary MLC (example Table I, experiments 5), while in the other four there was no difference in response to the two types of F_1 cells (even when there was a definitely stronger stimulation of DNA synthesis in the secondary response) (example Table I, experiments 6).

Analysis of "First Mitoses" in PHA-Stimulated Cultures and MLC.—Stimulation of B cells was shown in the above experiments by the predominance of BM mitoses on days 4 and 5, at a time when stimulation of DNA synthesis was still evident but no longer maximal. To study the time of appearance of the first wave of BM mitoses, cultures containing vinblastine sulfate (Velban 1×10^{-8} g/ml; Eli Lilly and Co., Indianapolis, Ind.) throughout the entire culture period were compared with normal cultures of the same chimeras. Arrest of cell division by vinblastine allows the study of mitoses of cells that have entered their first division in culture during the previous 8–12 hr, since dividing cells are arrested in metaphase and disintegrate after 8–12 hr (7). In a PHA-stimulated culture, the following results were observed: mitoses in the absence of vinblastine were 78% T on day 2 and 76% T on day 3; mitoses in a parallel culture performed with vinblastine were 74% T on day 2 and 37% T on day 3. In MLC (pooled results from several cultures) normal cultures showed 80% T mitoses on day 3, whereas parallel cultures in the presence of vinblastine showed 25% T mitoses (of 24 mitoses analyzed). Thus on day 3, both in PHA-stimulated cultures and MLC, while most mitoses are T, most cells entering for the first time into mitosis are B. This indicates that the first wave of B mitoses starts around day 3, when it is usually masked by the continuous proliferation of T cells. BM mitoses become increasingly evident in the subsequent days not only because of continuous B cell divisions but also probably as a result of progressive arrest of T cell divisions.

Response of T Cell-Depleted Spleen Cultures and of "In Vitro Chimeras".—In a large number of experiments, cultures of spleen cells depleted of T cells by treatment with rabbit anti-mouse-specific lymphocyte antigen (8) or mouse anti-θ + rabbit C showed no stimulation whatsoever by PHA or allogeneic cells in primary or secondary MLC. It was found that PHA responsiveness of T-depleted cultures could be restored by a very small number of T cells. As little as 8×10^4 cortisone-resistant thymocytes (an immunologically competent population of thymocytes) (9) of T6T6 mice, added to a culture of 1.5×10^6 CBA/Ca spleen cells depleted of T cells and thus unable to respond to PHA, allowed a definite response to PHA (stimulation index 8 on day 3). Karyotypic analysis of this in vitro type of "chimera" showed 65% T mitoses on day 2 and only 3% on day 3.

It thus appears, in agreement with recent evidence obtained in other systems of in vitro culture (10–13), that T cells stimulated by contact with PHA or allogeneic cells release a factor stimulating B cells. Two further points can now be added concerning this factor. First, its release does not seem to require completion of T cell mitoses, as indicated by its appearance in the supernatant of MLC within 24 hr (before appreciable enhancement of cell division), as well as by the experiments where mitoses were blocked by vinblastine. Second, the experiment reported in Table II indicates that the stimulating factor present in the supernatant of the 24 hr MLC is not a simple B cell mitogen. It does not

stimulate normal spleen cell cultures, but probably acts as a helper of B cells which have bound an agent unable by itself to induce their proliferation, such as PHA (14) as shown in the present experiments, confirming the results observed with concanavalin A by Andersson et al. (12). In MLC, B cell division might be triggered by the contact of some B cells with H-2 alloantigens in the presence of the helper factor released by the stimulated T cells. The early predominance of B cell mitoses in some secondary MLC might result from the presence in the spleen cell population of an expanded number of responsive B cells, rapidly induced to proliferate by "primed" T cells, whose own capacity to divide could be more limited than that of native T cells (15).

TABLE II

Effect of MLC Supernatant on DNA Synthesis in Cultures of Normal or "T-Depleted" Spleen Cells in the Presence of PHA

Normal C57 cell culture*	4200 cpm	C57 "T-depleted" cell culture*	2800 cpm
id. + control supernatant‡	2600 cpm	id. + PHA	2000 cpm
id. + MLC supernatant‡	4900 cpm	id. + PHA + control supernatant‡	2200 cpm
		id. + PHA + supernatant 24 hr MLC‡	32000 cpm

* 3 days' culture of 2×10^6 cells. T-depleted spleen cells are spleen cells treated with AKR anti-θC3H + rabbit C.

‡ Supernatant of 24 hr culture of 10×10^6 cells/ml, either of T6T6 alone (control supernatant) or of 5×10^6 T6T6 + 5×10^6 C57 (MLC supernatant). Cultures of normal and of T-depleted spleen cells were performed with 60% supernatant and 40% fresh medium.

SUMMARY

Spleen cell cultures of radiation chimeras (thymectomized, lethally irradiated mice repopulated with bone marrow cells and thymocytes bearing different chromosomal markers) were stimulated by phytohemagglutinin (PHA) and F_1 allogeneic spleen cells. Karyotypic analyses showed a marked predominance of T mitoses on the 2nd and 3rd days of culture followed by a strong predominance of B mitoses on the 4th and 5th days. Analysis of cells undergoing their first mitoses showed that the majority of T mitoses on day 3 resulted from continuous T cell division, and that most cells entering their first mitoses at that time were of B type. Mixed lymphocyte cultures (MLC) of chimeras immunized against allogeneic spleen cells showed sometimes, but not always, a response different from "primary" MLC, with an earlier and stronger predominance of BM mitoses. The role of stimulated T cells in the induction of B mitoses was shown by (*a*) the incapacity of T-depleted spleen cells to be stimulated by PHA or in primary or secondary MLC, and (*b*) the restoration of the mitotic response of B cells to PHA by adding to the T cell-depleted culture either a very small number of T cell (identified by their different karyotype: "in vitro chimeras") or the cell-free supernatant of a 24 hr MLC.

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